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Characteristics and *in Vitro* Fertilization Ability of Ram Spermatozoa: Comparison of Epididymal and Ejaculated Spermatozoa

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ABSTRAK

Penelitian ini dilakukan untuk mempelajari karakteristik spermatozoa dari kauda epididimis domba dan kemampuannya untuk memfertilisasi oosit secara *in vitro* setelah dibekukan. Spermatozoa dari ejakulat digunakan sebagai kelompok kontrol. Karakteristik (persentase motilitas, viabilitas, abnormalitas dan integritas membran) spermatozoa dari kauda epididimis dan ejakulat dievaluasi sebelum dan setelah dibekukan. Kemampuan spermatozoa untuk memfertilisasi oosit secara *in vitro* setelah dibekukan dievaluasi berdasarkan pembentukan pronukleus setelah fertilisasi *in vitro* (FIV). Data yang diperoleh pada penelitian ini menunjukkan bahwa karakteristik spermatozoa dari kauda epididimis segera setelah dikoleksi tidak berbeda dengan spermatozoa dari ejakulat. Motilitas spermatozoa kauda epididimis lebih rendah daripada spermatozoa ejakulat setelah equlibrasi ke-2 (65,00±3,54% vs 72,00±4,47%) dan setelah dibekukan (post-thawing) (48,00±4,47% vs 54,00±2,24%) (P<0,05). Namun demikian spermatozoa dari kauda epididimis mempunyai integritas membran yang lebih tinggi pada tahap *post-thawing* daripada spermatozoa ejakulat (75,38±9,32% vs 65,54±11,88%) (P<0,05). Berdasarkan pembentukan pronukleus setelah FIV didapatkan hasil bahwa tingkat fertilisasi spermatozoa dari kauda epididimis (61,40%, 42,98%, 18,42%, berturut-turut untuk total, normal, dan polispermia) tidak berbeda dengan spermatozoa ejakulat (66,67%, 48,78%, 17,89%, berturut-turut untuk total, normal, dan polispermia). Berdasarkan data di atas, dapat disimpulkan bahwa segera setelah dikoleksi, spermatozoa dari kauda epididimis mempunyai karakteristik yang sama dengan spermatozoa ejakulat. Walaupun setelah pembekuan motilitas spermatozoa kauda epididimis lebih rendah dari spermatozoa ejakulat, tingkat fertilisasi dari spermatozoa kauda epididimis tidak berbeda dengan spermatozoa ejakulat.

Kata kunci: spermatozoa, kauda epididimis, ejakulat, fertilisasi in vitro, domba

ABSTRACT

The characteristics and *in vitro* fertilization (IVF) ability of ram spermatozoa collected from cauda epididymal was examined. Ejaculated spermatozoa was used as control group in this experiment. Characteristics of spermatozoa including the percentage of progressive motility, viability, abnormality and membrane integrity were evaluated before and after freezing. Fertilization ability of post-thawed spermatozoa in both group was examined based on the pronucleus formation after IVF of *in vitro* matured (IVM) oocytes. Results from these study showed that there were no significant differences in the characteristics between cauda epididymal and ejaculated spermatozoa before freezing. After freezing, motility of ejaculated spermatozoa was higher than epididymal spermatozoa (54.00±2.24% vs 48.00±4.47%), however the membrane integrity of epididymal spermatozoa was higher than ejaculated spermatozoa (75.38±9.32% vs 65.54±11.88%) (P<0.05). The experiment revealed that the ability of post-thawed epididymal spermatozoa to fertilize oocytes (61.40%, 42.98%, 18.42% for total, normal and polysperm, respectively) did not differ from that of ejaculated spermatozoa (66.67%, 48.78%, 17.89% for total, normal and polysperm, respectively). These results indicate that ram spermatozoa collected from cauda epididymal and then frozen have the ability to fertilize ram ooctyes *in vitro* in the similar rate with ejaculated spermatozoa.

Key words: spermatozoa, cauda epididymal, ejaculate, in vitro fertilization, ram

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INTRODUCTION

Cryopreservation of semen has become a valuable tool for the preservation of genetic resources of endangered species or sires of superior breeding value. Semen cryopreservation offers many advantages to the livestock industry, particularly in conjunction with allowing the widespread dissemination of valuable genetic material even to small flocks by means of artificial insemination (AI). In addition to ejaculated spermatozoa, epididymal spermatozoa are also important candidate for cryopreservation, for examples if a genetic resource suddenly dies by accident or disease. Therefore, postmortem spermatozoa recovery is an useful strategy for germplasm banking (Tamayo *et al*., 2011). Spermatozoa stored in the cauda epididymal have usually good quality and a high level of maturation, being able to fertilize oocytes (Toshimori, 2003).

In Indonesia, sheep is a potential livestock for meat production. There are many slaugthered sheep have high genetic value. In order to preserve genetic resources after slaughtering, the cryopreservation of epididymal spermatozoa is an important method for the preservation of sheep genetic resource. To date, many studies have demonstrated that it is possible to obtain viable gametes postmortem. Furthermore, successful pregnancies have been achieved in many species using epididymal spermatozoa for artificial insemination (Hewit *et al*., 2001; Ikeda *et al*., 2002).

However, recovery of spermatozoa postmortem and their use has less attention in sheep than in other domestic animals (Ehling *et al*., 2006). After the death of animals, viability of spermatozoa is still high for a period of 10-20 h (Martinez-Pastor *et al*., 2005). Therefore, recovery of epididymal spermatozoa from dead animals, cryopreserved and subsequent utilization for in vitro fertilization (IVF) or AI are useful tools to rescue genetic material that otherwise would be lost, either from high genetic value animals or from endangered species (Santiago *et al*., 2006; Martins *et al*., 2007). Little information has been available concerning the characteristic of cauda epididymal spermatozoa in ram. Therefore, the objective of this study were to evaluate the characteristic and IVF ability of ram spermatozoa collected from cauda epididymal. As control, ejaculated spermatozoa was used in this experiment.

MATERIALS AND METHODS

Semen Collection and Cryopreservation

Ram testicles were obtained from slaughterhouse, and transported to the laboratory in physiological saline at room temperature. Cauda epididymal was removed from the testes and then sliced repeatedly with a scalpel blade to release spermatozoa in a culture dish containing 1 mL NSF-I extender. Fresh spermatozoa were colected from adult rams by artificial vagina. Characteristic of epididymal and ejaculated spermatozoa were evaluated before and after freezing for progressive motility, integrity plasma membrane, percentage of live spermatozoa (viability) and morphological abnormalities.

Cryopreservation of spermatozoa was performed according to the method described by Karja *et al*. (2002) with minor modifications. The suspension (about 500 µL) of spermatozoa was diluted with 450 µL of the first extender which consisted of 8.8% (w/v) lactose (Wako Pure Chemical, Osaka, Japan), 100 µg/mL penicillin and streptomycin and 20% (v:v) egg yolk in distilled water. The diluted spermatozoa were equilibrated in a refrigerator at 4 °C for 2 h. After equilibration, 250 µL of the second extender [the first extender supplemented with 6% (v:v) glycerol and 1.48% (v:v) orvus ES paste (Miyazaki Kagaku, Tokyo, Japan)] was added. The spermatozoa were then equilibrated at 4 °C for an additional 5 min. At the end of the equilibration period, the same volume (250 µL) of the second extender was added at 4 °C. The spermatozoa were immediately loaded into a 0.25 mL French straw (I.M.V., France). The spermatozoa were frozen by placing the straw on a styrofoam plate in liquid nitrogen vapor for 20 min (4 cm above the surface of liquid nitrogen), and subsequently stored in liquid nitrogen until examination. For Post-thawing evaluation, the straw was placed in air for 30 s, and then submerged into a 37 °C water bath for 30 s.

Spermatozoa Quality Assessment

Spermatozoa samples of each group was assessed for spermatozoa motility and viability before freezing and after thawing (frozen-thawed spermatozoa). Cryopreserved samples were thawed for 30 s in a 37 °C water bath. The percentage of motile spermatozoa was determined by microscopic examination on warm stage at 37 ºC at magnification of x400. Viability and morphology (percentage of total abnormal spermatozoa) were assessed by a supravital stain with eosin and nigrosin (Figure 1A). Examine using a bright field microscope (typically using a 40-100x objective lens). A total of 200 spermatozoa were analyzed for head, midpiece and tail normality spermatozoa with plasma membrane integrity was assessed by hypoosmotic swelling (HOS) test (Figure 1B).

In Vitro Fertilization

Frozen-thawed epididymal and ejaculated spermatozoa were washed in fertilization medium (Suzuki *et al*., 2000) by centrifugation at 1800 rpm, 37 °C for 5 min. The supernatant was removed and the spermatozoa pellet was diluted in fertilization medium until the spermatozoa concentration was adjusted to 5 x 10^6 spermatozoa/mL. After 28 h of in vitro maturation, oocytes were transferred separately into 100 µL of the spermatozoa microdrops (each with 10-15 cumulus-oocyte complexes) for fertilization and co-incubated for 14 h. After the co-incubation with spermatozoa, cumulus cells were removed mechanically by gentle pipetting. Oocytes were fixed, stained and then evaluated visually with an phase contrast microscope (Olympus IX 70, Japan) to confirm the fertilization by the presence of two or more pronucleus.

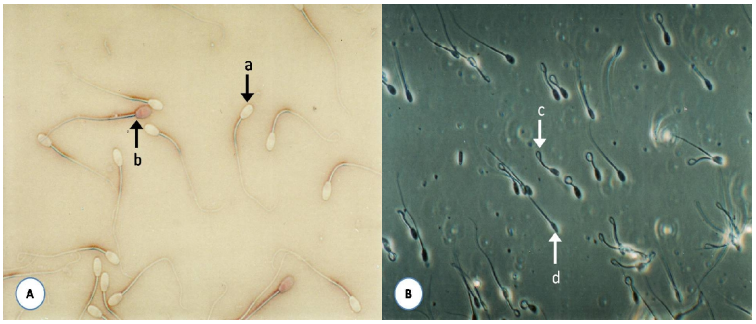

Figure 1. Assesment of spermatozoa (A); live spermatozoa with white spermatozoa head (a), dead spermatozoa with red spermatozoa head (b) (phase contrast microscope, 400x magnification), and (B) membrane integrity with HOS test; coiled spermatozoa (c) and not coiled (d) (phase contrast microscope, 200x magnification).

Statistical Analysis

Characteristic of spermatozoa and percentages of their fertilization ability in both groups were tested by ANOVA followed by Duncan's Multiple Range Test (DMRT) if ANOVA revealed a significant value.

RESULTS AND DISCUSSION

Characteristic of ram cauda epididymal spermatozoa before freezing were similar to ejaculated spermatozoa in this study (P>0.05). Following cryopreservation, the percentage of progressive motile, viability and membrane integrity decreased (Figure 2, 3, 4) in both groups, but morphologically abnormal rate of ram spermatozoa increased. Processing and storage cause changes to the spermatozoa membranes that are associated with loss of spermatozoa motility and fertility (Yeung *et al*., 2006). Cryopreservation of spermatozoa was reported to reduce acrosomal integrity in different species of animals (Rasul *et al*., 2001). The cryopreservation process includes several steps, to form spermatozoa preparation and dilution to the post-thawing maintenance of functional capability at each of these steps, spermatozoa can lose their ability to function normally. It has been reported that the motility of ejaculate spermatozoa is impaired in the course of cooling to 0 ºC (cold shock), freezing (ice formation and crystallization) and thawing (warm shock) (Watson, 2000). As a result of cooling, some workers have reported a decreased motility in spermatozoa. Rasul *et al*. (2001) reported a decreased motility and acrosomal integrity after thawing. Similar result was found in the present study.

The most significant cryoinjuries, in loss of cell integrity and cell death, resulting in a decrease in spermatozoa quality are sustained during the cryopreserva-

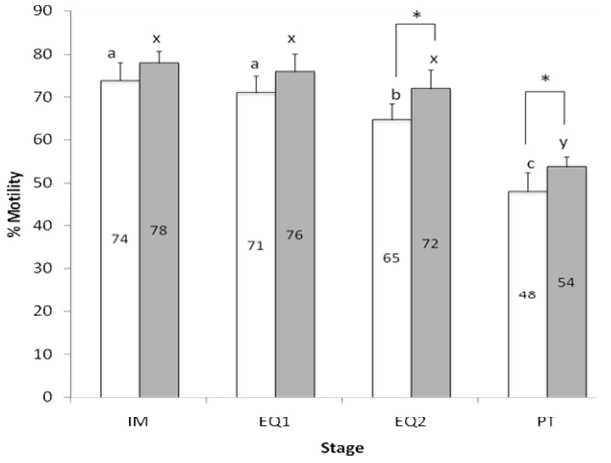

Figure 2. Percentage of progressive motility of pre-freezing and post-thawing from cauda epididymal (□) and ejaculated (■) ram spermatozoa. IM= immediately, EQ1= equlibration 1, EQ2= equilibration 2, PT= post thawing. Within each end point, bars with different letters (a, b; x, y) are significantly different for cauda and ejaculated spermatozoa (P<0.05).(*) There were significant differences (P<0.05) on motility within source of spermatozoa. The numbers on the bars represent the percentage of motility.

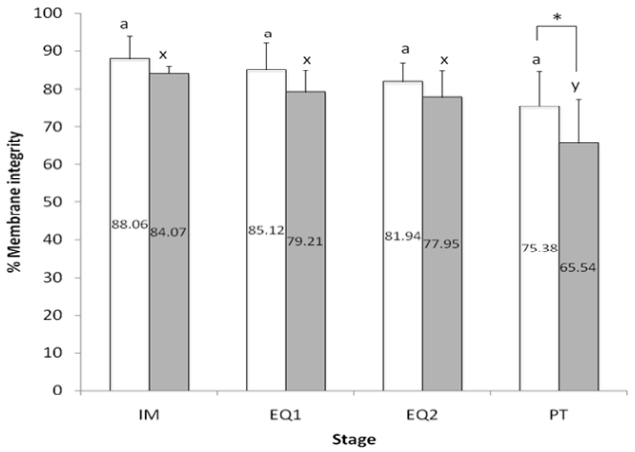

Figure 3. Percentage of membrane integrity of pre-freezing and post-thawing from cauda epididymal (□) and ejaculated (■) ram spermatozoa. IM= immediately, EQ1= equlibration 1, EQ2= equilibration 2, PT= post thawing. Within each end point, bars with different letters (a, b; x, y) are significantly different for cauda and ejaculated spermatozoa (P<0.05). (*)There were significant differences (P<0.05) on membrane integrity within source of spermatozoa. The numbers on the bars represent the percentage of membrane integrity.

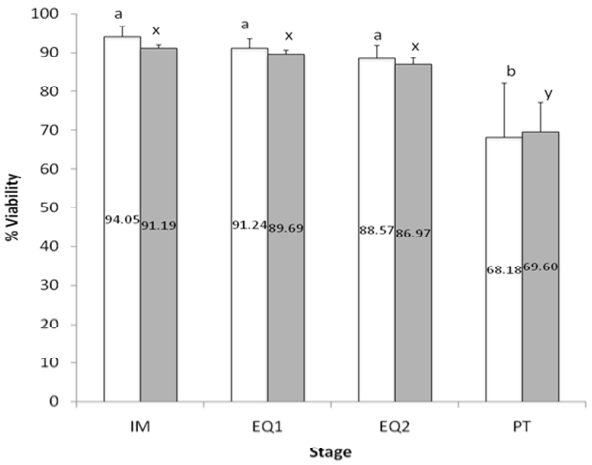

Figure 4. Percentage of viable spermatozoa of pre-freezing and post-thawing from cauda epididymal (□) and ejaculated (■) ram spermatozoa. IM= immediately, EQ1= equlibration 1, EQ2= equilibration 2, PT= post thawing. Within each end point, bars with different letters (a, b; x, y) are significantly different for cauda and ejaculated spermatozoa (P<0.05). The numbers on the bars represent the percentage of viable spermatozoa.

tion and thawing process as a result of induced lipid and protein re-arrangements within the cell membranes, osmotic stress across the plasma membranes, oxidative damage and intra- and extra-cellular ice damage (Watson, 2000). Rath & Niemann (1997) also recorded a decrease in post-thaw motility of boar cauda epididymal spermatozoa. Cold shock, a result of a rapid reduction in temperature from 25 to 4 °C, has been suggested as a cause for loss of post-thaw motility. Midpiece defects also resulting from cold shock have been implicated to negatively affect motility. Since the midpiece contains mitochondria responsible for oxidative metabolism, damage to the midpiece could affect energy flow to the flagella, thus lowering the post-thaw motility.

The percentage of progressive motile spermatozoa from cauda epididymal and ejaculated ram spermatozoa decreased after cryopreservation and thawing (Figure 2). After freezing, the motility of ejaculated spermatozoa was higher than that of epididymal spermatozoa (54.00±2.24% vs 48.00±4.47%) (P<0.05). A possible explanation for the higher spermatozoa motility in ejaculated spermatozoa is that the seminal plasma might have an activating effect on motility (Goovaerts *et al*., 2006). Spermatozoa, once differentiated in the testis, remain quiescent in epididymal fluids until they are released by ejaculation. Dilution of the epididymal fluid surrounding the spermatozoa, either with seminal plasma or with a semen extender, allows the initiation of spermatozoa motility and metabolism. But, the presence of immobilin in the cauda epididymis increases the viscosity of epididymal fluid and reduces the motility of spermatic cells (Garner *et al*., 2000), which substantiates findings in the present study. Hermansson & Axner (2007) reported that the epididymal sperm collection technique might also affect the spermatozoa quality as the sample would be contaminated by blood cells. The result were similar to those described by Hermansson & Axner (2007), who evaluated the susceptibility of feline ejaculated and epididymal spermatozoa to cold shock and found that ejaculated cat spermatozoa had better overall motility than epididymal spermatozoa. Tebet *et al*. (2006) reported that there was no significant differences between the electro-ejaculated and epididymal cat spermatozoa in terms of post-thaw motility. Rath & Neimann (1997) compared post-thaw motility of ejaculated and epididymal semen collected from three identical boars. Motility rates of epididymal spermatozoa were consistently better than those of ejaculated boar spermatozoa.

Spermatozoa react to hypo-osmomolality by developing bent or coiled tails. In this classic HOS test, this property is used to characterize membrane integrity. Bent tail morphology is a result of changes in the osmotic regulation in the spermatozoa. The spermatozoa tails wells and coils when exposed to hyperosmotic conditions, a sign that water transport across the membrane is occurring normally and the membrane has normal function which affect the metabolic processes to manage substrates and electrolytes transportation (Tartaglion & Ritta, 2004; Surachman *et al*., 2009). Bent tail in spermatozoa suggests possible temperature or osmotic shock effects and midpiece defects could be due to exposure to cold shock or hypotonic environment (Bissett & Bernard, 2005).

Plasma membrane integrity of the spermatozoa in cauda epididymal were higher than ejaculated samples (Figure 3). This is presumably because the spermatozoa from cauda epididymal have not been exposed to the secretion of sex gland accesories, where the secretion can modify the sensitivity of refrigeration and freezing resistance as in the spermatozoa from the ejaculate (Yu *et al*., 2002). At ejaculation processes, spermatozoa are mixed with seminal plasma where the content of plasma components that interact to modify the functions thus altering membrane properties and response of spermatozoa to the different treatments (Holt & Fazeli, 2010). The spermatozoa plasma membrane is rich in poly unsaturated fatty acids and is therefore susceptible to peroxidative damage with pH changes and consequent loss of membrane integrity, decreased spermatozoa motility, and eventually loss in fertility, resulting from reactive oxygen species during aerobic incubation (Yaniz *et al*., 2008).

As with other species in which semen preservation is routinely performance, the cooling of extended semen aids in preserving spermatozoa longevity by depressing of the spermatozoa cell's metabolic activity, which leads to significantly reduced energy consumption and by product formation. The cold-shock sensitivity is characterized by an irreversible loss of selective permeability and integrity of spermatozoa plasma membrane, all of which leads to perturbation and death of the cell (Robertson *et al*., 1990).

The reason for this higher resistance for epididymal ram spermatozoa to chilling stress may be attributed to higher phospholipid content to lower as spermatozoa matures during epididymal migration. The results in regard to damaged acrosomal integrity for ejaculated ram spermatozoa following hypothermic exposure are

in agreement with the Varisli *et al*. (2009) who found that cold shock induced reduction in acrosomal integrity and plasma membrane in ejaculated ram spermatozoa. Nevertheless, it was also found that epididymal boar spermatozoa were more cold-shock resistant than ejaculated spermatozoa which was consistent with the current data for ram spermatozoa. Interestingly, Gilmore *et al*. (1998) investigated cold-shock sensitivity of spermatozoa collected from several wildlife species and also found that electro-ejaculated spermatozoa from the worthhog, impala and elephant were more sensitive to cooling than epididymal spermatozoa. However, despite the fact that most of the spermatozoa were still have membrane intact, spermatozoa motile was greatly decreased.

There were no significant differences (P>0.05) between cauda epididymal and ejaculate samples for viability and morphological abnormalities in all parameters evaluated. Percentage of viability declined from 94.05±2.79% to 68.18±14.03% for cauda epididymal and 91.19±0.93% to 69.60±7.68% for ejaculate after cryopreservation and thawing (Figure 4). Whereas, percentage of abnormalities increased from 1.62±0.59% to 2.80±0.52% for cauda epididymal and 1.77±0.50% to 2.65±0.35% for ejaculate after cryopreservation and thawing. Following cryopreservation, percentage of viable spermatozoa decreased and morphologically abnormal of spermatozoa increased. It should be noted, however, that every epididymal spermatozoa fraction or ejaculate contains 1.62%–2.80% abnormal spermatozoa. These levels are considered acceptable and fertility may be reduced if morphological of abnormal spermatozoa exceeds 20% (Senger, 2005). Morphological changes have been attributed to spermatozoa response to changes in osmotic conditions. The response of cell volume from different species to different osmotic conditions reflects profound and different changes in the spermatozoa membranes. These changes are related to the permeability of the membrane to the major intracellular ions, functionality of ion channels and cytoskeletal integrity in different species (Petrunkina *et al*., 2005).

The *in vitro* binding assay was first described by Cramer *et al*. (1994) for fertility assessment. The *in vitro* penetration ability of spermatozoa after being frozen was evaluated after IVF experiments (Table 1). Figure 5 indicated the formation of pronucleus after IVF was performed. There were no significant differences between cauda epididymal and ejaculated spermatozoa (P>0.05) for the percentages of total, normal, and polyspermic fertilization. These data suggested that both cryopreserved, ejaculated and epididymal spermatozoa had a similar ability to fertilize oocytes *in vitro.* Spermatozoa from cauda epididymal have been reported has a similarity in the maturation index, the consistency of chromatin level and binding capability to zona pellucida proteins with ejaculated spermatozoa (Harkema *et al*., 2004; Garcia *et al*., 2006; Alvarez *et al*., 2009).

In this study, it was found that 17%-18% of oocytes were fertilized by more than one spermatozoa (P>0.05). Polyspermy, referring to fertilization involving more

Table 1. Number of oocytes fertilized in *in vitro* fertilization of sheep oocytes using cryopreserved spermatozoa

Spermatozoa	No. oocyte examined	No. (%) of oocytes fertilized				Fertilization rate (%)
		Not formed PN	Only 1PN	Normal 2PN	Polyspermic >2PN	
Cauda epididymal	114	32 (28,07)	12 (10,53)	49 (42,98)	21 (18,42)	70 (61,40)
Ejaculated	123	29 (23,57)	12 (9,76)	60 (48,78)	22 (17,89)	82 (66,67)

Note : PN= Pronucleus

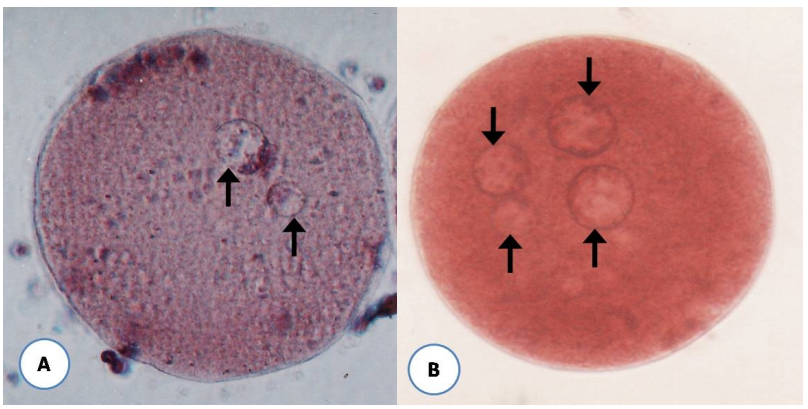

Figure 5. Pronucleus development of ewe oocyte after *in vitro* fertilization. A. two pronucleus (2PN), B. more than two pronucleus (>2PN). (Phase contrast microscope, 400x magnification).

than one spermatozoa, is one of the most commonly observed abnormalities in fertilization, generally resulting in embryonic death and one of the major factors affecting overall efficiency of IVF (Wang *et al*., 2003). Polyspermy is a pathological phenomenon that leads to developmental failure in various mammalian species. There are several mechanisms that exist within the female reproductive tract which regulate the number and quality of spermatozoa in order to reduce the risk of polyspermy (Hunter, 1996). However, in standard IVF methodologies, both male and female gametes are co-cultured in a medium drop, with no existing barriers regulating spermatozoa–oocyte interaction. Typically within this IVF system, large numbers of spermatozoa are present at the site of fertilization, often resulting in high numbers of spermatozoa penetrating the oocyte simultaneously, despite the fact that the oocyte itself has mechanisms to block the further penetration of spermatozoa after fertilization (Suzuki *et al.,* 2003). Therefore, a suboptimal IVF conditions is one of the reasons affecting polyspermic penetration.

CONCLUSION

The characteristic of ram cauda epididymal spermatozoa was similar to ejaculated spermatozoa. Ram spermatozoa collected from cauda epididymal and then frozen have the ability to fertilize ooctyes *in vitro*. Further work needs to be performed to investigate the uses of epididymal spermatozoa for artificial insemination.

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